Isolation of Coconut Storage Proteins by Polyacrylamide-Gel Electrophoresis

ROBERT W. WALLACE[1] AND JULIUS W. DIECKERT[2]

Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, The Texas A&M University System, College Station, Texas 77843

Received February 27, 1976; accepted May 25, 1976

By combining sodium dodecyl sulfate (SDS)-gel electrophoresis with a new chilling technique for visualization of protein–SDS complexes in polyacrylamide gels, a process has been developed which will permit the isolation of milligram quantities of pure polypeptides. Using this technique, we have isolated two molecular weight classes of polypeptides from coconut storage globulins and determined the amino acid composition of each. When the two amino acid compositions were summed on a molar basis, the result agreed reasonably well with the amino acid composition of the starting material with the exception of cystine. Apparently, some contaminant from the polyacrylamide caused its destruction to be accelerated during hydrolysis.

Since the introduction of electrophoresis of proteins after denaturation with sodium dodecyl sulfate (SDS) (1), the technique has been recognized as a powerful analytical tool and is used extensively to determine the molecular weight of polypeptides (2,3). Denaturation and wrapping of a protein with SDS, while often necessary when working with proteins insoluble in dilute buffers, severely limits the possibilities for further fractionation. Two possibilities are hydroxylapatite chromatography (4) and procedures which fractionate on the basis of size such as gel permeation chromatography and gel electrophoresis. The high resolving power of gel electrophoresis makes the technique attractive as a preparative procedure. Disadvantages of the technique are low capacity and a problem of removing ultraviolet-absorbing, nondialyzable contaminants from the acrylamide extracts.

Previous attempts at preparative electrophoresis (5) have resulted in the isolation of nanomole quantities of pure polypeptides. This is sufficient for characterization studies if microprocedures are used for N-terminal analysis, amino acid analysis, and tryptic mapping. Another approach (6) is to cut out stained bands from polyacrylamide gels and hydrolyze gel, dye, and protein to determine the amino acid composition. The very large quantity of ammonia released during hydrolysis

[1] To be included as a portion of a dissertation submitted by RWW for the Doctor of Philosophy degree from Texas A&M University.

[2] Author to whom correspondence should be addressed.

interferes with amino acid analyzers which use ninhydrin. This often results in spreading of the ammonia peak to a degree which prevents the quantitation of the adjacent histidine peak. High concentrations of ammonia will also form a precipitate with ninhydrin and stop the flow through the heating coils of the amino acid analyzer. In addition, the presence of gel components during hydrolysis may alter the hydrolysis rate or cause the destruction of various amino acids.

The techniques described in this paper permit the isolation of milligram quantities of polypeptides which allows many characterization studies to be conducted without resorting to microprocedures. The protein is eluted from the gel. Consequently, most of the gel components have been removed prior to hydrolysis. The technique involves electrophoresis on 102 × 3 × 90-mm polyacrylamide slabs, chilling of the slabs to visualize the protein bands as described previously (7), cutting the bands from the slab, and extracting the protein–SDS complexes.

MATERIALS

Coconuts were grown in Jamaica and donated by the Texas A&M Food Protein Research and Development Center. Bromophenol blue and reagent grade potassium persulfate were obtained from J. T. Baker Chemical Co. Acrylamide, *N*-*N'*-methylene-bis-acrylamide (Bis), and Coomassie brilliant blue R-250 were electrophoresis grade from Bio-Rad Laboratories. 2-Mercaptoethanol and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Eastman Organic Chemicals. Sodium dodecyl sulfate (SDS) and 4 N methanesulfonic acid with 0.2% 3-(2-aminoethyl)-indole were Sequanal grade from Pierce Chemical Co. Ammonium sulfate was enzyme grade from Schwarz/Mann. Preparative electrophoresis was performed in an Ortec Model 2100 electrophoresis apparatus.

METHODS

Preparation of Coconut Storage Proteins

Isolation of the S2-45-45 coconut isolate is summarized in Fig. 1. The white coconut endosperm from mature coconuts was first extracted by mixing in a Waring Blendor with 0.1 M NaCl, pH 7.0, 50 mM sodium phosphate buffer which was 0.02% in sodium azide (buffer A). After centrifugation, three layers were present: a top layer of solid coconut fat, a middle layer of solvent with dissolved coconut protein, and a precipitate on the bottom. The top two layers were removed, and the precipitate was extracted in a Waring Blendor with 1.0 M NaCl, pH 7.0, 50 mM sodium phosphate buffer (buffer B). The slurry was centrifuged, and the supernatant was brought to 45% saturation with ammonium sulfate. After

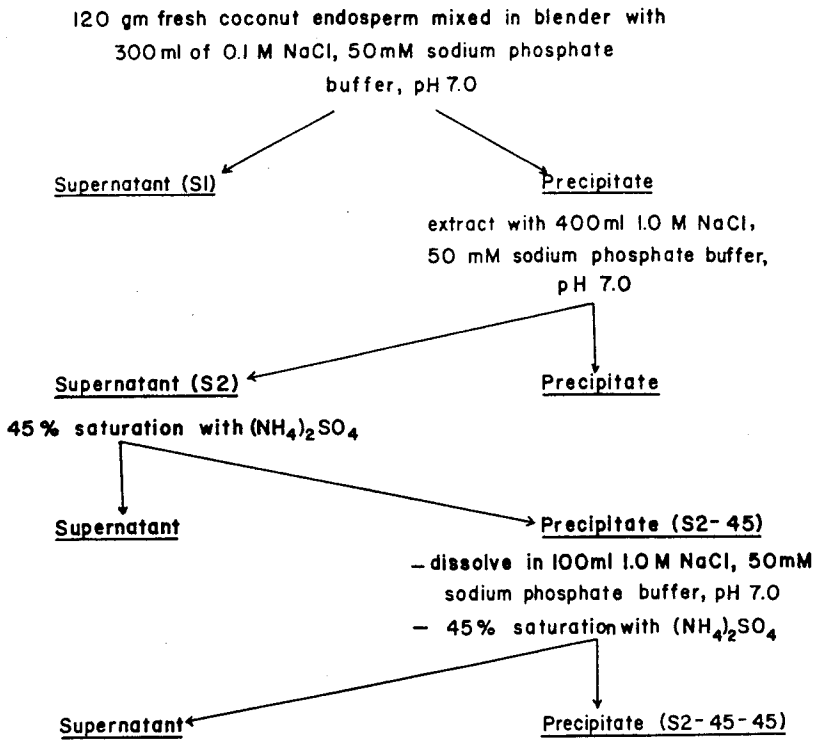

FIG. 1. Isolation of the S-2-45-45 fraction of coconut globulins.

stirring for 2 hr at room temperature, the slurry was centrifuged and the precipitate redissolved in buffer B. The protein solution was again brought to 45% saturation with ammonium sulfate. The resulting precipitate was centrifuged, dissolved in buffer B, and dialyzed for 48 hr against the same buffer at 9°C. This protein isolate was labeled S2-45-45 and is the starting material for preparative electrophoresis.

Electrophoresis

The electrophoresis system is essentially the same as that described by Weber and Osborn (2) with a few modifications. The S2-45-45 protein solution was combined with sufficient 10% SDS in water to provide five times as much SDS as protein on a weight basis. The mixture was immediately placed in a 100°C water bath and incubated for 5 min. It was then transferred to a dialysis bag and dialyzed against distilled water for 48 hr. The desalted protein solution was freeze-dried and dissolved in sufficient 10 mM sodium phosphate buffer, pH 7.2, to give a 2% protein solution. A portion of this solution was subjected to analytical SDS electrophoresis, and the molecular weights of the peptides were determined according to the procedure described by Weber and Osborn (2). The relative proportion of each polypeptide was determined by scanning the Coomassie blue-stained gel at 650 nm in a Gilford Model 240 spectrophotometer.

The acrylamide gels for preparative work were 7% acrylamide, of which 3% was Bis (7% T, 3% C). They are prepared by combining 12.5 ml of 28% T, 3% C acrylamide stock solution, 25 ml of gel buffer (0.2 M sodium phosphate buffer, pH 7.2, 1.0% SDS, 0.04% sodium azide), 0.075 ml of TEMED, and 10 ml of water. The solution is mixed well in an ice bath and then 2.5 ml of potassium persulfate solution (10 mg/ml) is added. The acrylamide solution (25 ml) is delivered into each of two Ortec slab-gel molds. The acrylamide solution is layered with sufficient isobutyl alcohol, as described by Neville (8), to provide a flat interface between the acrylamide and the alcohol. The acrylamide should polymerize within 10 to 15 min. Then the isobutyl alcohol is removed and the gel surface is washed with distilled water and layered with a 1:1 dilution of gel buffer and water. The gels should be allowed to polymerize for 1 to 2 hr before they are used for electrophoresis. They may be stored for several weeks at 4°C without noticeable changes in their electrophoretic properties.

The amount of protein applied to the gel will vary depending upon the degree of separation of the protein bands of interest. The two predominant polypeptides in the S2-45-45 coconut isolate are sufficiently separated to allow 5 mg of protein to be applied to the total surface area of the gel. The 2% S2-45-45 protein–SDS solution (0.25 ml) is combined with seven to eight crystals of sucrose, 10 μl of a 0.5% solution of bromophenol blue tracking dye, and 10 μl of 2-mercaptoethanol. The solution is incubated for 5 min at 100°C. We have found that it is best to fill both the upper and lower reservoir buffers of the electrophoresis apparatus before the protein is applied. The protein solution can then be layered on top of the acrylamide gels with a Pasteur pipet.

Electrophoresis was for 4 to 5 hr with a current of 20 mA/cm^2 of gel surface area (163 mA for the Ortec system) with the anode in the bottom chamber. During electrophoresis the upper chamber pH rises rapidly. This problem can be controlled by changing the upper electrode buffer twice during the 3- to 4-hr electrophoresis period. The lower electrode buffer is routinely used for three runs before it is discarded. In preparative work it is important not to mix the upper and lower reservoir buffers since charged impurities such as persulfate (9–11), which are removed from the gel during the initial electrophoresis, will be reintroduced into subsequent gels after the buffers are mixed and will possibly cause artifacts in the banding patterns.

After electrophoresis, the protein bands can be visualized by chilling as described previously (7). The gels are removed from the casting molds and placed on a glass chromatography plate. They are covered with a layer of Saran Wrap and chilled overnight at 4°C or for several hours on cracked ice. The protein bands appear as white opaque areas in the gel. They may then be removed by cutting the gel with a razor blade.

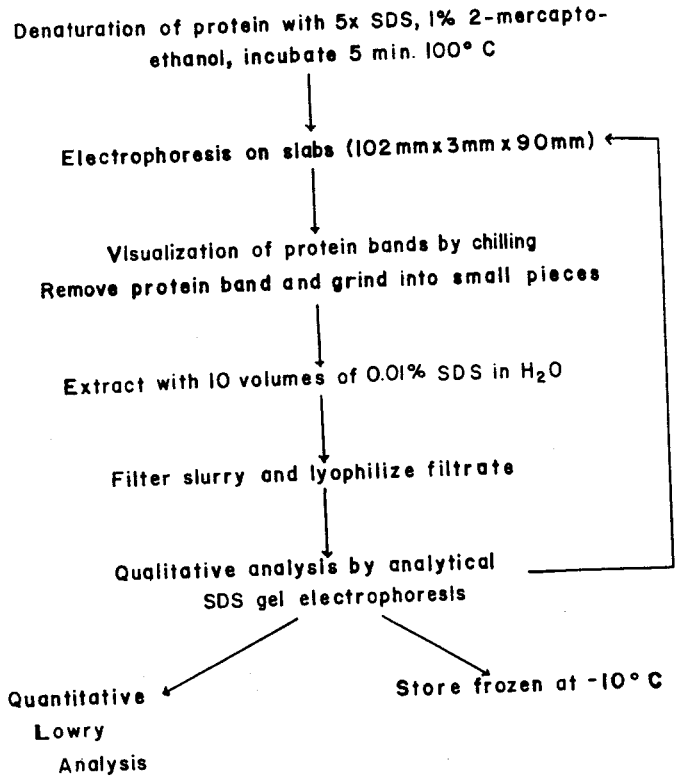

FIG. 2. Strategy for isolation of SDS–peptides by preparative electrophoresis.

and the gel containing the desired protein band is macerated by the action of a glass stirring rod on the side of a beaker containing the gel. Twenty or more volumes of 0.01% SDS in water are applied, and the slurry is stirred overnight at room temperature. The slurry is filtered through Whatman No. 41 filter paper and then through a 0.45-μm Millipore filter. The supernatant is concentrated by freeze-drying.

The protein extracted from the acrylamide gels was analyzed qualitatively by Weber and Osborn's procedure of analytical gel electrophoresis (2). Quantitative analysis for protein was by the procedure of Lowry *et al.* (12) after the protein had been precipitated with 10 vol of 10% perchloric acid, 1% phosphotungstic acid.

If the product is not of desired quality, it may be recycled through the electrophoresis procedure as shown in Fig. 2. We have been able to purify proteins which have very similar mobilities with a maximum of two cycles of electrophoresis. With the S2-45-45 coconut isolate, only one cycle of electrophoresis was necessary.

Amino Acid Analysis

Hydrolysis of the polypeptides was with methanesulfonic acid according to a modification of the procedure of Liu and Chang (13): (i) A protein sample containing a minimum of 3.6 mg of protein dissolved in SDS is

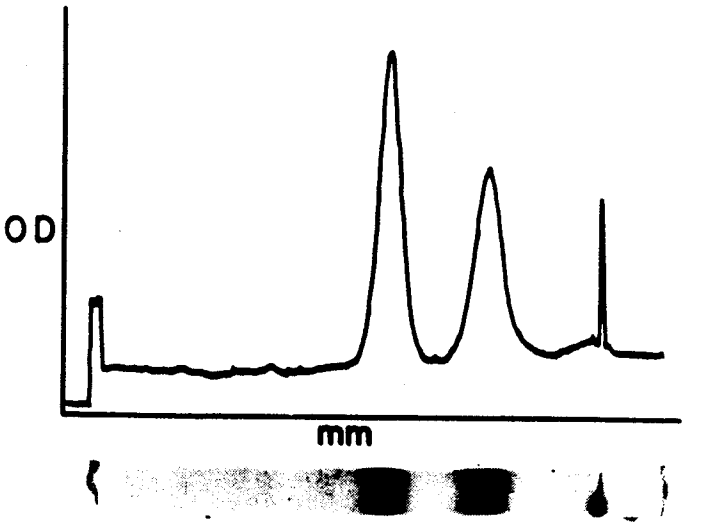

FIG. 3. Quantitative scan of S2-45-45 coconut isolate. Optical density measured at 650 nm on a Gilford Model 240 spectrophotometer.

dialyzed overnight against distilled water. (ii) The protein solution is removed from the dialysis bag and precipitated by the addition of 10 vol of acetone. Then it is redissolved in a sufficient quantity of 10% SDS in water to give a concentration of 8–15 mg/ml. (iii) A portion of this solution containing 0.8–1.5 mg of protein is transferred with a 100-μl Hamilton syringe to each of three 1-ml ampules. (iv) Sufficient acetone is added to the ampule to make the total volume 1 ml. The ampule is placed in an ice bath for 1 hr and then centrifuged in a clinical centrifuge. The white protein precipitate packs well on the bottom of the ampule, and the supernatant can be removed with a Pasteur pipet. The precipitate is dried thoroughly over a stream of nitrogen. (v) Methanesulfonic acid (4 N, 0.5 ml) containing 0.2% 3-(2-aminoethyl)-indole is added to the ampule. (vi) The ampule is alternately evacuated with a water aspirator and flushed with nitrogen. This is continued for four or five cycles. Finally, the ampule is evacuated for 10 min and sealed under vacuum with an oxygen torch. (vii) The ampules are heated for periods of 24, 48, and 72 hr at 110°C. (viii) After hydrolysis, the content of each ampule is removed quantitatively, combined with 0.5 ml of 3.5 N NaOH, and diluted to a final volume of 1.5 ml. (ix) Duplicate aliquots of 0.25 ml are applied to the long and short columns of a Beckman 121C amino acid analyzer. (x) The amino acid data are plotted as a function of hydrolysis time. The average values for three hydrolysis times, the values obtained by extrapolation to zero hydrolysis time, or the 72-hr data were used as considered appropriate. (xi) The amino acid composition data are compared by calculating the difference indices as described by Metzger *et al.* (14) and Dieckert and Dieckert (15).

TABLE 1

DATA ON FOUR PREPARATIVE ELECTROPHORESIS EXPERIMENTS

Experiment	Total protein Electrophoresed (mg/slab × slabs)	Total protein Extracted from gel (mg)			Recovery (%)
		P_{51}	P_{30}	P_{19}	
I	1 × 2	—	0.63	0.32	73
II	2 × 2	—	1.07	0.63	65
III	3 × 2	0.244	1.31	0.97	63.5
IV	5 × 2	0.439	2.32	1.54	52.9

RESULTS AND DISCUSSION

The procedure for the isolation of the S2-45-45 coconut protein is similar to that for cocosin as described by Khaund (16) and most likely contains the same proteins. The SDS-electrophoresis pattern and quantitative scan of the gel is shown in Fig. 3. The isolate is dominated by two molecular weight classes of polypeptides with molecular weights of 30,000 and 19,000 as determined by SDS-gel electrophoresis. In addition, a small amount of polypeptide with a molecular weight of 51,000 was present. The polypeptides were labeled P_{30}, P_{19}, and P_{51}, respectively. The S2-45-45 isolate accounts for 27.6% of the total protein extracted from the coconut.

It is tempting to speculate that the P_{30} and P_{19} polypeptides combine to form a 49,000-dalton subunit in the native molecule. Integration of the quantitative scan of the Coomassie blue-stained gel gives the relative proportions of P_{19}:P_{30} to be 1:1.26. If they were present in exactly equimolar amounts, the result should be 1:1.58. The difference in the observed and expected relative proportions may be due to the inaccuracies inherent in the molecular weight estimates or due to difficulties in scanning Coomassie blue-stained gels, (17) or there might be a small amount of contaminating protein which has a molecular weight of approximately 19,000.

During preparative electrophoresis, the pH of the upper electrode buffer, as well as the gel, will rise to as high as 10.5 to 11.0 if the upper reservoir buffer is not changed during the run. This was first observed when a strong smell of ammonia was detected from the gel after a run. Apparently, the pH had risen high enough to hydrolyze the amide groups of the polyacrylamide. In addition to this problem, disulfide interchange was observed in certain polypeptides with cystine residues. It seems likely that breaking of certain peptide bonds which are highly susceptible to basic hydrolysis could also take place. The increase in pH would also be expected to cleave some of the amides in the asparagine and glutamine residues of the proteins. This would introduce charge heterogeneity and complex patterns if the purified polypeptides were later

FIG. 4. Qualitative analysis of preparative electrophoresis Experiment IV (Table 1). Starting material is the S2-45-45 coconut isolate.

checked for purity by a procedure such as isoelectric focusing. Changing the upper reservoir buffer solves the problem, but a better solution would be to design an electrophoresis apparatus which has a large buffer reservoir at both the upper and lower electrode. The Ortec design provides a 3-liter lower electrode buffer chamber, but the upper chamber will hold only 0.75 liter of buffer.

The P_{19} and P_{30} bands are well separated with 5 mg of protein placed on the gel. Even the P_{51} polypeptide is visible after chilling. There was no problem in cutting the polyacrylamide slabs before the SDS–protein complexes redissolved and disappeared. However, if a large number of bands is being removed from a complex pattern, this may become a problem. It is possible to visualize the protein bands again by rechilling the gels without noticeable changes in the patterns, provided the gels have not been left at room temperature for a long period of time.

TABLE 2

AMINO ACID COMPOSITION OF THE S2-45-45 COCONUT ISOLATE AND THE 19,000- AND 30,000-DALTON POLYPEPTIDES

Amino acid	Protein isolate				
	P_{19}	P_{30}	$P_{19} + P_{30}$	S2-45-45	Δmol%
Lys	4.10	2.09	2.91	2.48	0.43
His	1.04	1.14	1.10	1.22	0.12
Arg	11.35	12.37	11.96	11.89	0.07
Trp	0.363	0.427	0.401	0.407	0.006
Asp	9.41	9.70	9.59	9.24	0.35
Thr	5.04	3.83	4.32	4.46	0.14
Ser	7.48	8.01	7.80	7.24	0.56
Glu	12.89	19.59	16.88	16.56	0.32
Pro	4.27	4.37	4.33	4.43	0.10
Gly	8.44	7.89	8.11	7.98	0.13
Ala	7.18	5.84	6.38	6.30	0.08
½-Cys	0.265	0.803	0.565	1.72	1.16
Val	7.65	6.18	6.77	6.74	0.03
Met	2.13	0.994	1.45	2.15	0.70
Ile	5.45	3.21	4.12	4.11	0.01
Leu	6.90	7.31	7.15	6.91	0.24
Tyr	2.62	2.44	2.51	2.55	0.04
Phe	3.43	3.81	3.66	3.53	0.13
D.I.[a]					2.31

[a] Difference index (D.I.) is calculated according to Metzger (11).

Table 1 summarizes the quantitative data from four electrophoretic isolation experiments. One of the major problems of the preparative electrophoresis technique is the extraction of the protein from the gel. In these experiments, the recovery was 53–73%, and the yield decreased as the amount of protein applied to the gel increased. Figure 4 shows the result of the qualitative analysis of Experiment IV of Table 1.

The amino acid compositions of the S2-45-45 isolate, P_{30}, and P_{19} are shown in Table 2. The $P_{19} + P_{30}$ data were computed by summing the analysis of P_{19} and P_{30} on a molar basis. Calculation of the difference index (D.I.) provides a means of comparing the amino acid composition of two proteins (14,15). The procedure is to express the amino acid composition of the two proteins as mole percent (mol%). Then use the following formula to calculate the difference index:

$$\text{D.I.} = \tfrac{1}{2}\Sigma(\Delta\text{mol}\%).$$

If the two amino acid compositions are identical, the difference index will be zero. However, if the two proteins have no amino acids in common, the result will be 100. Our experience has indicated that a

difference index as high as 1.5 or 2.0 may be obtained due to experimental error when duplicate analyses of the same proteins are conducted. When the $P_{19} + P_{30}$ values are compared with the amino acid composition of the S2-45-45 isolate, a difference index of 2.31 is obtained. Upon further inspection, it is noted that the largest deviation for any amino acid is for cystine with a Δmol% of 1.16. If the cystine data are subtracted, the difference index drops to 1.73. This indicates that with the exception of cystine it is possible to obtain reliable amino acid composition data, even with contaminants from the polyacrylamide gel present during hydrolysis. One possible explanation for the large deviation in the cystine values is that a contaminant from the polyacrylamide gels increased the rate of destruction of cystine during hydrolysis.

When the proteins which had been extracted from acrylamide gels were hydrolyzed and analyzed with the amino acid analyzer, a very large ammonia peak was present. However, the procedure described in this paper resulted in an ammonia peak which remained on scale and did not result in peak spreading or the formation of a precipitate in the heating coils of the amino acid analyzer as has been reported by those who hydrolyze the polyacrylamide gel with the protein (6). Apparently, soluble acrylamide from the gel was responsible for the ammonia peak. We are now experimenting with a number of possible procedures for the removal of this material from the extracted protein prior to hydrolysis.

ACKNOWLEDGMENTS

We would like to thank Mrs. Billie B. Hefti of the Texas A&M Food Protein Research and Development Center for her assistance in determining the amino acid compositions of the isolates. This work was supported by the following contracts to J. W. Dieckert: Cotton Incorporated Cooperative Agreement No. 75-682, Agency for International Development Contract No. AID-csd-2804, and Texas Agricultural Experiment Station Project H-1306.

REFERENCES

1. Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28,** 815.
2. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406.
3. Dunker, A. K., and Rueckert, R. R. (1969) *J. Biol. Chem.* **244,** 5074.
4. Moss, B., and Rosenblum, E. N. (1972) *J. Biol. Chem.* **247,** 5194.
5. Weiner, A. M., Platt, T., and Weber, K., *J. Biol. Chem.* **247,** 3242.
6. Stein, S., Chang, C. H., Bohlen, P., Imai, K., and Udenfriend, S. (1974) *Anal. Biochem.* **60,** 272.
7. Wallace, R. W., Yu, P. H., Dieckert, J. P., and Dieckert, J. W. (1974) *Anal. Biochem.* **61,** 86.
8. Neville, D. M. (1971) *J. Biol. Chem.* **246,** 6328.
9. King, E. E. (1970) *J. Chromatogr.* **53,** 559.
10. Brewer, J. M. (1967) *Science* **156,** 256.
11. Fantes, K. H., and Furminger, I. G. S. (1967) *Nature (London)* **215,** 750.

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
13. Liu, T. Y., and Chang, Y. H. (1971) *J. Biol. Chem.* **246,** 2842.
14. Metzger, H., Shapiro, M. B., Mosimann, J. E., and Vinton, J. E. (1968) *Nature (London)* **219,** 1168.
15. Dieckert, J. W., and Dieckert, M. C. (1976) *J. Food Sci.* **41,** 475.
16. Khaund, R. N. (1971), Ph.D. Dissertation, Texas A&M University, College Station, Tex.
17. Fisbein, W. N. (1972) *Anal. Biochem.* **46,** 388.